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APPENDIX A

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Biochemical Interactions at the Endothelium

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CHAPTER 5

Glycoproteins secreted by the endothelium and their involvement in specific interactions at the subendothelium

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CONTENTS

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1. Introduction

This chapter will consider the synthesis and secretion into the extracellular space, by endothelium (primarily vascular endothelium), of a range of glycoproteins. In general, these glycoproteins may be regarded as constituents of, or as occurring in, the endothelial matrix and/or to function within the subendothelial environment. In particular, extensive reference will be made to collagens, fibronectin, basement membrane glycoproteins including laminin and, to a lesser extent, entactin and amyloid P-component, and the plasma glycoprotein Factor VIII/von Willebrand factor. Their participation in certain important interactions at the subendothelial surface will be discussed, especially their possible involvement in the interaction of platelets with the vascular subendothelium and in the attachment of the endothelium itself to its underlying substratum. Although not a glycoprotein, brief mention will be made of elastin together with its associated microfibrillar glycoprotein as constituents of the elastic fibre, a recognised component of the vascular subendothelium. This chapter will not attempt to review endothelial glycoproteins secreted as enzymes, such as plasminogen activator or angiotensin converting enzyme nor, in general, glycoproteins that may be regarded more as cell membrane con-

stituents. Complementary to the interests of this chapter, basement membrane structure is reviewed in detail in Chapter 3 and some aspects of the influence of the extracellular matrix on endothelial function are considered in Chapter 13.

2. Glycoproteins of the endothelium

2.1. Collagens

The existence of several collagenous species as members of a closely related but nevertheless genetically distinct group of proteins is now well recognised (Bornstein and Sage, 1980). Various different collagen types occur in the blood vessel wall where they play an essential structural role. They also serve to initiate, through their ability to induce the aggregation of blood platelets, formation of the haemostatic plug, the development of which is necessary for the arrest of bleeding following injury (Barnes, 1982). Arising also from their interaction with platelets, collagens are able to promote the complex sequence of reactions resulting in coagulation, which culminates in the formation of fibrin. The latter serves to reinforce or stabilise the platelet aggregate as well as acting as the basis for coagulation. Bearing in mind their involvement in haemostasis, collagens in the vessel wall may also be regarded as potentially important causative agents in the initiation of thrombosis, which can be regarded as the pathological expression of the haemostatic mechanism (Thomas 1977, 1978; Nordoy, 1979; Wall and Harker, 1980).

2.1.1. Collagens of the blood vessel wall

Following the initial observations of Chung and Miller (1974), several authors have reported the presence of the two interstitial collagens, types I and III, in the blood vessel wall and have commented on the relative abundance of type III in this tissue (Trelstad, 1974; Epstein and Munderloh, 1975; Gay et al., 1975; McCullagh and Balian, 1975; Rauterberg and Bassewitz, 1975; Barnes et al., 1976a; Scott et al., 1977). A number of reviews of the general properties and distribution of these two collagens and their alterations in disease has been presented (e.g. Miller, 1976; Ramachandran and Reddi, 1976; Gay and Miller, 1978; Prockop et al., 1979; Bailey and Etherington, 1980; Bornstein and Sage, 1980; Eyre, 1980; Minor, 1980; Jayson and Weiss, 1982) and, therefore, a detailed description of each collagen is not necessary here. The type I collagen molecule of chain composition $[\alpha 1(I)]_2\alpha 2$ differs in the primary structure of its constituent α-chains from the type III collagen molecule of chain composition $[\alpha 1(III)]_3$. Molecules of each collagen, however,

aggregate in the same highly ordered fashion to yield the characteristic striated fibre of 67 nm periodicity, as observed under the electron microscope. Both collagens are presumed to occur as fibres of this nature within the entire width of the tunica media of the vessel wall. Type III appears to be closely associated with the elastic laminae, whilst type I is located more in the spaces in between these structures (Gay et al., 1975; 1976; McCullagh et al., 1980). The smooth muscle cell can be regarded as the cell of origin for both collagens in the media (Barnes et al., 1976b; Leung et al., 1976; Burke et al., 1977; Layman et al., 1977; Rauterberg et al., 1977; Scott et al., 1977; Mayne et al., 1977, 1978) whilst in the adventitia the adventitial fibroblast may be presumbed to serve as their source (Mayne et al., 1977). In the young vessel wall, where little if any intimal thickening is likely to have occurred and the tunica intima can be regarded as constituting little more than endothelium, collagen type III has been detected by immunofluorescence, apparently in the total absence of type I, in the space between the endothelium and the underlying internal elastic lamina (Gay et al., 1975, 1976). This has led to the proposal that type III may be a particularly important collagen in relation to thrombosis. As discussed in the following section it is conceivable that type III collagen in this location is a direct product of the endothelium. Immunofluorescence studies have also tended to emphasise the relative abundance of this collagen type in the diffusely thickened intima of the older vessel wall (McCullagh et al., 1980). However, biochemical analysis has demonstrated that type I collagen is the predominant species in thickened intima, as in the media and adventitia (Morton and Barnes, 1982). Presumably the collagens deposited in the thickening intima are mostly the products of smooth muscle cells that have migrated from the media into the intimal space.

Similarly, the deposition of collagen, predominantly type I, with lesser amounts of type III, in the intimal atherosclerotic plaque (McCullagh and Balian, 1975; Morton and Barnes, 1982) can be attributed to invading smooth muscle cells derived from the media (Ross and Glomset, 1976). In view of the presence of both these collagens in diffusely thickened intima, as well as in the atherosclerotic plaque, we consider injury to the intimal surface of the vessel wall will just as readily expose fibres of type I as of type III especially since the former represents the predominant species. Because fibres of each type are equally effective at aggregating platelets (Barnes et al., 1976a) both collagens can be regarded as of potential importance in the initiation of thrombosis that arises from platelet interaction with the subendothelium.

The basement membrane collagen, type IV, has been isolated from intact human and bovine aorta. Although it can be obtained as a partially purified product from pepsin digests, by salt fractionation and other techniques (Trels-

tad, 1974; Mayne et al., 1980), it has not been isolated specifically from intima. The presence of a specific collagen type in basement membranes was first indicated by chemical analysis of these structures following their isolation from various sources and subsequently confirmed by its widespread occurrence throughout all basement membranes as deduced from localisation studies utilising immunofluorescent techniques. This collagen, which is believed to play an important role in the filtration and other specialised properties of basement membranes has been intensively studied in a number of laboratories in recent years and the subject is reviewed in detail elsewhere in this book in the chapter by V.C. Duance and A.J. Bailey on the structure and function of vascular basement membranes (Chapter 3). Immunofluorescence studies have confirmed the presence of type IV in the blood vessel wall where it appears to be located both within the basement membranes of medial smooth muscle cells (Gay et al., 1981b) and in the subendothelium, as a constituent of the basement membrane immediately underlying the endothelium, from which it is, in this case, presumably derived (Madri et al., 1980a).

Collagen type V, regarded by some as closely associated with basement membranes (even if not actually a genuine constituent of them), is a collagen about which there is still much controversy, particularly concerning its distribution and structure. It has been described by Gay et al., (1981a, 1981b) as a pericellular collagen fulfilling an essentially exocytoskeletal role. Three constituent polypeptide chains designated as A-, B- and C-chains (or α2(V)-, α1(V)- and α3(V)-chains, respectively) have been described, but the precise relationship between these with regard to the chain composition of the type V collagen molecule is unclear. It is conceivable that there is a family of type V collagens based on various combinations of the three chain types. This collagen is discussed more fully elsewhere (see Chapter 3). In relation to its occurrence in the blood vessel wall, Chung et al. (1976) have detected the presence of a type V collagen containing solely B-chains in the media of human aorta. No chains of type V origin were detected in the intima. By contrast, Morton and Barnes (1982) have found chains of both A and B-type (in a ratio of B : A in the region of 1.5) throughout the vessel wall, in intima, media and adventitia and have noted them to be particularly abundant in the atherosclerotic plaque. This observation has also been made by Ooshima (1981), although this author reported a much higher ratio of B : A-chains (7.6). In pig aorta, but not human, a trace of the C-chain constituent has also been found (Morton and Barnes, 1982). Chains of A- and B-type have also been isolated from bovine aorta (Mayne et al., 1980). Immunofluorescence studies have confirmed the presence of collagen type V in both intima and media of the vessel wall (Madri et al., 1980a; McCullagh et al., 1980) where, in the media, it appears to be intimately

associated with the basement membrane of the smooth muscle cell (Gay et al., 1981b; Martinez-Hernandez et al., 1982). In the subendothelial location collagen type V is also thought to be of basement membrane origin and, as such, a product of the endothelium (Madri et al., 1980a). These authors have concluded that it also resides on the endothelial cell surface.

In addition to collagens I, III, IV and V, a further collagenous species has been detected in the intima of human aorta and in highly vascular tissues such as human and bovine placenta (Chung et al., 1976; Furuto and Miller, 1980; Jander et al., 1981). This collagen, referred to as 'short-chain' collagen, can be solubilised by pepsin digestion as a high molecular weight aggregate that upon reduction yields chains with a molecular weight of 55000 in the case of the constituent of aortic intimal origin or, in the case of that of placental origin, 40000. This makes this material very much smaller than the normal constituent collagen constituent α-chain size of 95000. Chung et al. (1976) believe this species may also be of basement membrane in origin. The site of synthesis of this collagen is as yet unclear but its derivation from endothelium seems a reasonable conjecture. Finally, the subendothelium may also contain another collagenous element, designated 'endothelial' collagen that has so far only been detected in endothelial cell cultures (Sage et al., 1980) and the identification of which is referred to in the following section.

2.1.2. Collagen synthesis by endothelium

2.1.2.1. Vascular endothelial cells. A major product of the endothelium (as of epithelium) is thought to be the underlying basement membrane with which it is in intimate contact. From this it might be considered that the main collagenous component synthesised by endothelium would be collagen type IV, previously recognised as the specific collagenous moiety of basement membranes. In accord with this supposition, Howard et al. (1976) have reported that calf aortic endothelial cells in culture synthesise predominantly collagen of basement membrane type. Collagenase-sensitive material produced by these cells was found to contain a relatively high content of 3-hydroxyproline (representing approximately 10% of the total hydroxyproline) and glucosylgalactosylhydroxylysine (constituting approximately 90% of the total glycosylated residues and about 70% of the total hydroxylysine), both features being considered characteristic of collagen type IV. Collagen in the medium from endothelial cell cultures was identified essentially as a single component which, after reduction, migrated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a molecular weight of approximately 135000 and was considered to be procollagen α1(IV)-chains. Similarly, from a study of human endothelial cells derived from umbilical cord veins, Jaffe et al. (1976) con-

cluded that these cells synthesised material in culture that could be identified by electron microscopy as resembling amorphous basement membrane. This material reacted with an anti-human glomerurar basement membrane antiserum. Biosynthetic studies led to the identification in the cell layer, following pepsin treatment, of two collagenous components, the major one of which, had a molecular weight of 120 500 and was considered to be the α1(IV)-chain. A second, more minor component, of molecular weight 94 200, was tentatively thought to correspond to α1(III)-chains. In the culture medium three collagenous components were recognised after pepsin digestion, one of molecular weight 183 000 postulated to be a β-dimer of the α1(III)chain, one of 152 000 considered to be the α1(IV) procollagen chain and a third of 115 000 proposed as α1(IV)-chains. These authors concluded that their endothelial cells synthesised basement membrane collagen as a major collagenous product. Madri et al. (1980a) have also reported that confluent cultures of human umbilical vein and calf aortic endothelial cells can be stained by immunofluorescence or immunoperoxidase procedures using antibodies against collagens types IV and V, but not with those against types I and III. Immunoprecipitation of collagens, using monospecific collagen antibodies, confirmed the synthesis in culture of types IV and V that were located entirely in the cell layer and furthermore suggested the absence of any synthesis of collagens types I and III.

In contrast to these findings Barnes et al. (1978) concluded that porcine aortic endothelial cells in culture synthesised collagen that was principally of interstitial type. In initial studies this was found to be largely type I in nature with some type III and type I trimer. Collagens were identified in pepsin digests on the basis of their precipitation behaviour and according to their positions of elution during ion-exchange chromatography and gel filtration. In subsequent studies it was found that synthesis could vary from largely type I, with little type III, to almost entirely type III with little if any type I. The synthesis of interstitial collagen was accompanied by the production of only relatively small amounts of collagens types IV and V (Sankey and Barnes, 1980; Sankey, 1981). In these studies collagens were separated, following pepsin digestion, by the salt fractionation procedure described by Kresina and Miller (1979) and identified by SDS-PAGE. Type V collagen was identified as a mixture of A- (or α2(V)-) and B- (or α1(V)-) chains; type IV was tentatively identified as a collagenous component occurring in the appropriate fraction that, upon reduction, migrated as a species of molecular weight around 140 000. Essentially similar results to these have been described by Sage et al. (1979, 1981) studying bovine aortic endothelial cells. They have concluded that these cells synthesize predominantly type III collagen most of which is secreted into the medium. Type III was identified by a number of criteria, including

specific immunoprecipitation and the nature of the cleavage products obtained by treatment with either mast cell protease or cyanogen bromide. Small amounts of type V-derived A and B-chains and of collagen type IV, identified as a polypeptide of molecular weight 140 000 following reduction, were found in the cell layer. Synthesis of type I collagen was only noted in those cultures which exhibited 'sprouting', a phenomenon observed as a secondary growth pattern in which variant endothelial cells, so-called 'sprouting' cells, can be seen to undergrow the normal endothelial monolayer. Synthesis of type I has also been attributed to these 'sprouting' cells by Cotta-Pereira and co-authors (1980). However, the present authors and their colleagues have not observed any pattern of collagen synthesis that can be related obviously to the extent to which 'sprouting cells' occur within a culture.

Reasons for these differences as regards the nature of the principal collagen synthesised by cultured vascular endothelial cells are at present unclear. They seem unlikely to be simply a consequence of subcultivation since we have obtained essentially identical results in primary and secondary cultures. Where collagens have only been very tentatively identified it is conceivable that this identification was in error. Sage et al. (1981) have suggested, for example, that the two collagenous components detected in cell layers by Jaffe et al. (1976) were probably A- and B-chains of type V collagen. The identification of type III collagen as a major product of endothelium in culture is of particular interest in view of the immunolocalisation of this collagen in the absence of type I in the subendothelial region of the young blood vessel wall (Gay et al., 1975, 1976).

Sage et al. (1980) have reported, in addition to the synthesis of collagens types III, IV and V, the synthesis by bovine aortic endothelial cells of a novel collagen, which they have designated 'endothelial' collagen. This particular collagen species is extremely sensitive to pepsin, being rapidly degraded to peptides of less than 60 000 molecular weight. It has been identified in the culture medium as a non-reducible species of molecular weight 177 000 together with two lower molecular weight species of 125 000 and 100 000 believed to originate from the higher molecular weight form. Examination of peptides obtained by treatment with mast cell protease or cyanogen bromide has confirmed the novel nature of this collagen.

2.1.2.2. Corneal endothelial cells. A similar ambiguity to that which occurs with regard to the nature of the principal collagen produced by vascular endothelial cells in culture exists in respect of endothelial cells of non-vascular origin, i.e. those from the cornea. A number of authors have presented evidence for the synthesis of predominantly collagen type IV by rabbit corneal endothelial cells in culture, a finding perhaps not unexpected in view of the

close apposition of these cells to a well-defined basement membrane (Descemet's membrane), (Kefalides et al., 1976; Sundar Raj et al., 1979; Kenney et al., 1981). In contrast studies by the authors and their colleagues (Sankey et al., 1981) have shown that corneal endothelial cells of bovine origin synthesise predominantly collagen type III, some type I and a small amount of type V with the absence of any species recognisable as type IV (see Fig 5.1). Essentially similar results have been reported by Tseng et al. (1981) although these authors detected a small amount of type IV collagen. We have observed the same pattern of synthesis for rapidly dividing cells, cells maintained at confluence for extended periods, cells grown on plastic, or on type I or type IV collagen-coated surfaces.

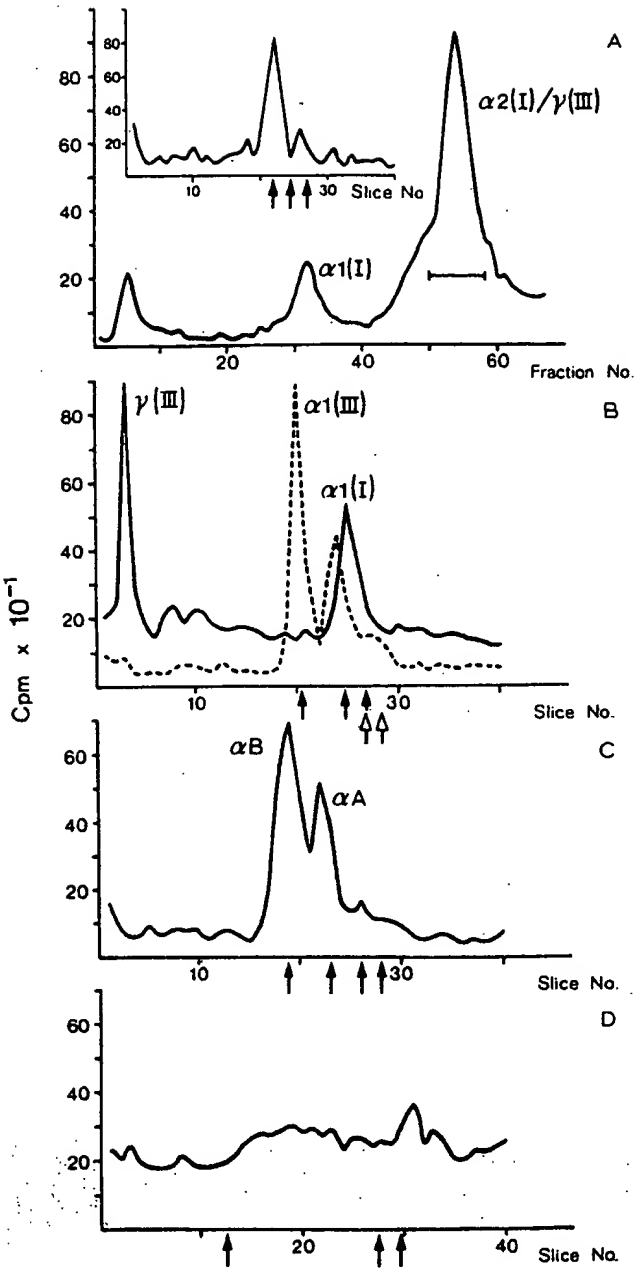
We consider the factors controlling the nature of the collagenous products of the endothelium and their level of synthesis an important issue, not only in terms of platelet activation and its relation to haemostasis or thrombosis, but also in the context of endothelial integrity. They are equally of importance in the processes of vessel wall repair including re-endothelialisation and the deposition of new subendothelium and in the pathogenesis of other vascular disease other than thrombosis. The latter, for example, may be illustrated by the thickening of vascular basement membrane that occurs in diabetic microangiopathy. The role of subendothelial collagens in the stimulation of platelets and their possible involvement in the attachment of the cells of the endothelium to its underlying substratum, an important factor in the maintenance of endothelial integrity, will each be considered later in this chapter.

2.2. Fibronectin

Fibronectin is a high molecular weight glycoprotein which occurs in a soluble form in plasma (Mosesson and Umfleet, 1970) and other body fluids (Chen et al., 1976a; Kuusela et al., 1978), and in an insoluble form associated with certain basement membranes (Bray, 1978; Stenman and Vaheri, 1978), in loose connective tissue matrices and in the pericellular matrix around adherent cultured cells (reviewed by Yamada and Olden, 1978; Ruoslahti et al., 1981b).

2.2.1. Distribution

Although fibronectin is often found at the interface of basement membranes and abutting endothelial (or epithelial) cells (Oberly et al., 1979), and possibly within these and other limiting membranes (Linder et al., 1975), there is some controversy as to whether this glycoprotein is a genuine component of these matrices. Most of the studies indicating the localisation of fibronectin within the basement membrane have been with adult mammalian tissues and have utilised fluorescent antibody localisation techniques. These studies have indi-


A
α2(I)/γ(III)
α1(I)
Slice No
Fraction No.
B
γ(III)
α1(III)
α1(I)
Cpm x 10⁻¹
Slice No.
C
αB
αA
Slice No.
D
Slice No.

cated the presence of fibronectin within the subendothelial basement membrane of a variety of blood vessels, around the smooth muscle cells of the media of muscular arteries and as thin strands in the media of non-muscular arteries and loose connective tissue of the adventitia (Stenman and Vaheri, 1978). Linder et al. (1975) have also shown the presence of fibronectin in the subendothelium of arteries, veins, brain capillaries and the renal basal membrane of chick embryos. However, little or none of the glycoprotein has been shown in the basement membranes of normal mature mammalian kidney, with the fibronectin that was demonstrable being associated mainly with the cell surface of the mesangium (Stenman and Vaheri, 1978; Petterson and Colvin, 1978; Oberley et al., 1979; Weiss et al., 1979; Burns et al., 1980; Dixon et al., 1980; Martinez-Hernandez et al., 1981). Biochemical studies have also indicated the close association of fibronectin and basement membranes although these studies cannot eliminate the possibility that this fibronectin is plasma fibronectin entrapped within the membrane matrix (Oh et al., 1981).

2.2.2. *Structure*

Although plasma and cellular fibronectins appear to share a common structural organisation they are not identical in some details of their structure, chemical composition and biological activities. Fibronectin is a large dimeric molecule of approximate molecular weight 450 000 composed of two sub-units, each of molecular weight about 220 000 linked by disulphide bonds (Mosher, 1980a) and which are structurally indistinguishable by peptide mapping

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Figure 5.1. Analysis of collagen types synthesized by cultured bovine corneal endothelial cells

Radiolabelled cultures, following pepsin digestion, were precipitated with 0.7 M NaCl (for collagen types I and III) and 1.2 M NaCl (for collagens IV and V). The latter precipitate was further processed to yield separate 'IV' and 'V' fractions. (A) CM-cellulose chromatography of the fraction precipitated at 0.7 M NaCl. The position of elution of collagen chains as indicated was established by chromatography of appropriate standards. Fractions under the bar were combined and examined by interrupted electrophoresis (inset) which confirmed the presence in this peak of α2(I) and γ(III) chains. The arrows denote in turn the position of the collagen marker chains α1(III), α1(I) and α2. (B) Gel electrophoresis of the 0.7 M NaCl-precipitated fraction. Direct electrophoresis (continuous line) shows the presence of γ(III) and Type I chains. The arrows (↑) denote the positions in turn of α1(I) and α2 marker chains. Interrupted electrophoresis (broken line) shows the conversion of the γ-component to α1(III) chains. The arrows (↑) denote in sequence the positions of α1(III), α1(I) and α2 markers. (C) Gel electrophoresis of the 'type V' fraction showing the presence of αA and αB chains of type V collagen. The arrows indicate the positions of αB, αA, α1(I) and α2 markers respectively. (D) Gel electrophoresis of the 'type IV' fraction after reduction. The arrows indicate the position of β, α1(I) and α2 markers. It was calculated that in this experiment collagens types I, III and V were synthesized in the proportion of 2:6:1. Reproduced with permission from Sankey et al., 1981 (*Biochem. J.* 198, 707–710).

(Kurkinen et al., 1980). When analysed on SDS-PAGE the polypeptide chains of plasma fibronectin separate into two bands (Mosesson et al., 1975), although cellular fibronectin generally produces only a single band which runs at a position corresponding to the slower of the two plasma fibronectin bands (Hynes et al., 1978; Quaroni et al., 1978). However, the mobility of fibronectin derived from different cell types of the same organism may also show some variability (Engvall et al., 1978; Yamada and Kennedy, 1979; Crouch et al., 1978; Alitalo et al., 1980b; Ruoslahti et al., 1981a). It has been suggested that these differences may be partially due to differences in the degree of glycosylation of these molecules and indeed plasma fibronectin contains considerably less fucose than cellular fibronectin (Fukada and Hakomori, 1979; Takasaki et al., 1979; Ruoslahti et al., 1981a) and also differs from cellular fibronectin in the linkages of bound sugars, although the total sugar content was similar in both fibronectin species (Mosesson et al., 1975; Vuento et al., 1977; Yamada et al., 1977).

Fibronectin is strongly antigenic and with conventional antibodies identical reactivity is shown against plasma and cellular forms from the same species (Ruoslahti et al., 1973; Ruoslahti and Engvall, 1978). However, Atherton and Hynes (1981) have obtained monoclonal antibodies that distinguish cellular from plasma fibronectin. These monoclonal antibody specificities did not appear to be due to differences in carbohydrate side chains because they precipitated fibronectin from cells cultured in either the absence (glycosylated) or presence (non-glycosylated) of tunicamycin equally well. This observation, together with the report of at least 3 regions of polypeptide difference between plasma and cellular fibronectin functional domains (Hayashi and Yamada, 1981), indicates that the two molecules are probably coded by independent genes or that there are different splicing mechanisms for producing tissue specific fibronectins.

2.2.3. Fibronectin structure and molecular interactions

Partial cleavage of fibronectin by various proteolytic enzymes has been useful in the structural and functional characterisation of this protein. Using this technique, specific domains have been assigned on the monomeric form of fibronectin, although the arrangement of these domains is still tentative. Three main regions have been identified; (a) the basic NH_2-terminus area, which contains binding sites available to FXIIIa (plasma transglutaminase), *Staphylococcus aureus* and fibrin and which mediates FXIIIa catalysed cross linking to collagen, (b) the non-covalent collagen (gelatin)-binding area, which is adjacent to this region and, (c) the CO_2H terminal region which is essentially responsible for heparin and eukaryotic cell attachment. Fibronectin has also

been shown to interact with DNA, actin, fibrinogen, hyaluronate, heparan sulphate and gangliosides at specific sites. These data have been recently reviewed by Ruoslahti, 1981; Ruoslahti et al., 1981b; Mosher and Furcht, 1981 and Hayashi and Yamada, 1981.

The interaction of fibronectin with collagen has been studied in some detail. Fibronectin has been shown to bind more effectively to denatured than to native collagens. Of the native collagens type III is more effectively bound than native collagen types I, II or V (Jilek and Hormann, 1978; Engvall et al., 1978). So far no comparisons are available of the interaction of fibronectin with fibres composed of the different collagen types, although Kleinmann et al. (1981) have shown that fibronectin binds more effectively to forming than to preformed fibres. Studies with fragments of collagen chains indicate that there are a number of fibronectin binding sites along the collagen polypeptide chains (Dessau et al., 1978a; Engvall et al., 1978) although Kleinman et al. (1978a) concluded that the major binding site for fibronectin was situated at residues 757–791 (which includes the mammalian collagenase cleavage site) on the α-1(I) chain. Dessau et al. (1978a) have also shown that residues 693–1101 of the α-2 chain of type I collagen, a region homologous to the binding site of the α-1(I) chain, form a major binding site for this chain. The possible role of the collagen-fibronectin interaction in the sub-endothelium will be considered later in this chapter, in the sections dealing with the collagen-platelet interaction (Section 3.1.2.1.) and cell-matrix interactions relevant to endothelial attachment (Section 3.2.).

2.2.4. Fibronectin synthesis

As mentioned earlier (Section 2.2.1.) fibronectin has been shown to be located in close proximity to endothelial cells and to be present in the subendothelium of a variety of blood vessels (Linder et al., 1975; Vaheri et al., 1977; Stenman and Vaheri, 1978), and consequently it has been suggested that these cells may be the major site for plasma fibronectin production. This is supported by the observation that endothelial cells in culture have been shown to synthesise relatively large amounts of fibronectin. Jaffe and Mosher (1978) have demonstrated that cultured human umbilical vein endothelial cells synthesised fibronectin, which could be secreted into the medium or incorporated into the subcellular matrix. The medium fibronectin comprised about 15% of the secreted proteins of these cells and was present at levels comparable to those in the plasma. Macarak et al. (1978) and Birdwell et al. (1978) have also shown that bovine aortic endothelial cells produced similar or higher levels of fibronectin. Birdwell et al. (1978) using immunofluorescence techniques, examined in some detail the cellular distribution of fibronectin. In confluent

cultures fibronectin was only detected in the subcellular matrix but was apparent on the dorsal surfaces of cells at regions of cell-cell contact in sparse cultures. These cells were thus similar to fibroblasts in that they produced a subcellular fibronectin matrix, but differed in that they did not produce a fibrillar fibronectin matrix on the dorsal cell surface (Chen et al., 1976b; Wartiovaara et al., 1974; Mautner and Hynes, 1977). This incorporation of fibronectin into the subcellular matrix in the absence of any deposition on the dorsal endothelial surface may be an instance of a phenomenon exhibited by other secreted endothelial products and as such may conceivably be of significance in the maintenance of the non-thrombogenic nature of the endothelium. The synthesis of fibronectin has also been demonstrated in a wide variety of differentiated cell types including those of epithelial origin (Chen et al., 1977b; Quaroni et al., 1978; Crouch et al., 1978; Voss et al., 1979; Smith et al., 1979; Foidart et al., 1980b) which, in that fibronectin was found only in a sub-cellular location, were similar to endothelial cells.

2.2.5. Functions of fibronectin

A number of apparently diverse functional properties have been proposed for fibronectin, based on its structural properties and extensive distribution in tissue and body fluids. Studies of the interaction of fibronectin with collagen and eukaryotic cells have indicated strongly that one of the major roles of fibronectin is that of mediating the binding of cells to the underlying tissue collagens. It is not intended to discuss this particular role of fibronectin here as it will be dealt with later in Section 3.2. but to consider the additional roles that have been attributed to fibronectin and which may be relevant to its function in the subendothelium.

As a plasma protein, fibronectin has been implicated in the blood coagulation process since it is capable of being enzymatically cross-linked to fibrin by factor XIIIa. In fact, approximately 3–4% of the normal clot is fibronectin (Mosher, 1980b; Chen et al., 1977a). Consequently, it has been suggested that fibronectin may regulate the rate and degree of fibrin monomer aggregation, its binding with factor XIIIa ensuring a high local enzyme concentration for the cross-linking of fibrin and fibronectin in clot formation. It has also been shown that fibronectin and its plasmin degradation fragments enhance the urokinase activation of plasminogen and indeed Iwanaga et al. (1978) have suggested that fibronectin may directly affect the lysis rate of a thrombus. The ability of fibronectin to bind and precipitate fibrinogen with heparin also suggests a further possible role in the coagulation process (Stathakis and Mosesson, 1979). Fibronectin may also be involved in primary haemostasis

and its possible role in platelet adhesion and aggregation will be discussed in detail in Section 3.1.2.1.

Plasma fibronectin has been shown to be identical to α-2-opsonic glycoprotein, which has been shown to promote the uptake of gelatin coated particles by liver slices (Blumenstock et al., 1977) and their uptake and phagocytosis by peritoneal macrophages (Doran et al., 1980; Gudewicz et al., 1980; Marquette et al., 1981; van de Water et al., 1981). Phagocytosis of *Staphylococcus* by leukocytes is also stimulated by fibronectin (Mosher, 1980b). Such data suggest that plasma fibronectin may act as an opsonin, acting to bind and coat circulating bacteria, tissue fragments or microscopic blood clots, which may then be removed by the reticulo-endothelial system.

Fibronectin has been postulated both as a promoter and as an inhibitor of cell motility. For example, fibronectin has been shown to confer properties likely to enhance cell locomotion upon transformed cells (Yamada et al., 1976; Ali et al., 1977), and to increase the motility of certain cell lines (Pouyssegur et al., 1977; Ali and Hynes 1978). Schor et al. (1981) however, have shown that fibronectin acts not only as an inhibitor of the migration of human skin fibroblasts into three-dimensional collagen gel matrices, but also as a stimulator of melanoma cell migration into the same matrices. Couchman and Rees (1979) also showed that cessation of fibroblast migration from chick heart explants was correlated with the appearance of fibronectin at the cell surface. The mechanism by which fibronectin may affect cell migration is unknown but it has been suggested that it may be due to changes in the continuity of the filamentous elements of the cytoskeleton and fibronectin via transmembrane linkages (Hynes and Destree, 1978; Singer, 1979). Evidence for the involvement of fibronectin in cell movement may indicate a possible role for this protein in endothelial cell migration, a process which may be important in the repair of damaged endothelium (cf. Chapter 13).

Various other functions have been attributed to fibronectin including (a) the direction of differentiation (Chen, 1977; Podleski et al., 1979; Dessau et al., 1978b; Ruoslahti et al., 1981b; Pennypacker et al., 1979; West et al., 1979; Thesleff et al., 1979; Wartiovaara et al., 1978; Zetter et al., 1978); (b) the direction of morphogenic movements in embryonic development (Linder et al., 1975; Wartiovaara et al., 1978; Zetter et al., 1978; Critchley et al., 1979; Mayer et al., 1980); and (c) as a chemoattractant for fibroblasts (Tsukamoto et al., 1981; Seppa et al., 1981), which has been shown (Postlethwaite et al., 1981) to be confined to a major 140000 fragment generated by cathepsin D cleavage. The large number of very diverse functions which have been attributed to fibronectin together with its widespread distribution probably demonstrates the considerable physiological importance of this glycoprotein.

2.3. *Laminin*

2.3.1. *Structure*

Laminin is a large non-collagenous glycoprotein, structurally and immunologically unrelated to fibronectin, which was originally isolated from neutral salt extracts of the Engelbreth-Holm's Swarm (EHS) sarcoma, a mouse tumour that produces a considerable extracellular matrix of basement membrane-like material (Timpl et al., 1979). The laminin isolated from the matrix of this and similar tumours consists of a very high molecular weight (about 900 000), disulphide linked complex of three polypeptide chains, the largest of which has a molecular weight of 450 000 with the two additional chains each being of molecular weight about 230 000 (Chung et al., 1979; Sakashita and Ruoslahti, 1980; Cooper et al., 1981). Antibodies to laminin also precipitate a fourth polypeptide of molecular weight 150 000 which is made in greater amounts by normal rather than tumorigenic endoderm cells (Hogan et al., 1980), although the relationship of this protein to authentic laminin is not yet clear. A more detailed description of the structure of laminin can be found in Chapter 3 of this volume.

2.3.2. *Distribution*

Laminin has been shown to be a major constituent of adult (Foidart et al., 1980a) and embryonic (Leivo et al., 1980; Howe and Salter, 1980; Hogan et al., 1980) basement membranes of presumed endothelial origin (Rhode et al., 1979; Hahn et al., 1980) and epithelial origin (Risteli and Timpl, 1981; Hogan et al., 1980; Smith and Strickland, 1981). Immunofluorescence studies using antiserum to EHS tumour-derived laminin have shown that this glycoprotein (or an immunologically related protein) occurs in a variety of basement membranes in normal tissue, including the basement membranes underlying the endothelial lining of the intima and around the smooth muscle cells of the large blood vessels (Rhode et al., 1979; Hahn et al., 1980). Laminin seems to be widely distributed and has also been demonstrated in the basement membranes of the glomerulus, tubules and Bowman's capsule of the kidney, lung alveolae, the choroid plexus of the brain, the lens capsule, chorionic and amniotic membranes, Descemet's membrane of the cornea, and the membrane surrounding muscle fibres (Rhode et al., 1979). Other authors have demonstrated the presence of laminin in the basement membrane of the portal tracts of normal liver, beneath the endothelial lining of hepatic arteries, portal veins and lymphatic vessels, and to be around smooth muscle cells in the vessel walls, bile ducts and ductules and nerve axons (Hahn et al., 1980). A more detailed ultrastructural study of laminin location in normal murine kidneys has shown

laminin to be found uniformly distributed throughout tubular basement membranes, the mesangial matrix and Bowmans capsule. In glomerular basement membranes however, laminin is restricted to the lamina rara interna and adjacent regions of the lamina densa (Madri et al., 1980b) and has also been shown to be restricted to the lamina rara of human epidermal and mouse oesophageal epithelial basement membranes (Foidart et al., 1980b). The presence of laminin has also been shown in Reichart's membrane, a thick membrane formed between the trophoblast and parietal endoderm cells of early mammalian embryos (Hogan et al., 1980; Smith and Strickland, 1981), and bovine lens capsule (Risteli and Timpl, 1981). Small amounts of laminin have also been detected in the circulation, presumably derived from the endothelial lining of the vascular wall (Risteli and Timpl, 1981; Risteli et al., 1980).

2.3.3. Synthesis of laminin

The synthesis and cellular location of laminin has been studied by immunoprecipitation and indirect immunofluorescence techniques in both vascular and corneal endothelial cells as a function of cell growth and organisation (Gospodarowicz et al., 1981). Both cell types were capable of secreting laminin into the incubation medium and of incorporating it into the subcellular matrix. Vascular endothelial cells produced nearly twenty times as much laminin in the medium (approximately 6% of the protein secreted into the medium) as corneal endothelial cells, although this may only be apparent because the latter cells secrete a more substantial extracellular matrix than the former and furthermore the insoluble laminin was not quantitated. Laminin secretion into the medium decreased with cell density (about four fold for vascular endothelial cells, compared with approximately thirtyfold for corneal endothelial cells), which may reflect a preferential accumulation of laminin within the extracellular matrix, because this reduction in secretion coincided with the production of such a matrix by these cells. Laminin was not detected at the apical cell surface in dense cultures although in sparse cultures it was detected at the extremity of filipodia in vascular endothelial cells and at the cell periphery in corneal endothelial cells.

The synthesis of laminin or a molecule of very similar mobility characteristics has also been demonstrated in a variety of other cell types, such as endodermal cells (Hogan et al., 1980; Smith and Strickland, 1980; Cooper et al., 1981; Sakashita and Ruoslahti, 1980), tumour cells (Howe and Salter, 1980; Strickland et al., 1980; Wewer et al., 1981; Alitalo et al., 1980a; Timpl et al., 1979), 3T3 cells, epithelial cells (Foidart et al., 1980b, Timpl et al., 1979) and myoblasts (Timpl et al., 1979). All these cell types are similar to endothelial cells in that the laminin they secreted into the incubation medium does

not, by itself, form an extensive fibrous matrix on the upper and lower surfaces of these cells but is incorporated into the subcellular matrix.

2.3.4. Function of laminin

The biological role of laminin is not known, although, it has neen suggested that it may be involved in functions as diverse as signalling cellular differentiation and growth, protein filtration and cell-substratum interactions. It has been proposed, for example, that laminin may be involved in early embryo development (Leivo et al., 1980) and in kidney tubule embryogenesis, being implicated in the increased adhesiveness of mesenchyme cells (Ekblom et al., 1980). It is, however, possible that in these cases laminin may be an early manifestation of epithelial differentiation although its assembly into a basement membrane may actively direct cell polarisation (Hogan, 1981). Should laminin indeed play a role in epithelial cell polarisation it is likely that it may also have a similar function for endothelial cells. As a glycoprotein containing high proportions of acidic residues and sialic acid, laminin has also been considered as one of the polyanions potentially capable of forming a charge barrier restricting the passage of plasma proteins across the glomerular basement membrane (Madri et al., 1980b), a membrane which has been shown to contain laminin (Timpl et al., 1979; Chung et al., 1979) but not fibronectin (Bray, 1978). Little is known of the function of Reichart's Membrane, another basement membrane shown to contain laminin but little fibronectin (Hogan, 1980), but it is thought that it may act as a passively selective filter for substances passing from the maternal to the foetal environment (Jollie, 1968). Thus a high laminin to fibronectin ratio may be a feature of basement membranes the function of which is to act primarily as molecular filters rather than as sites for cell attachment and migration. The likely involvement of laminin as a cell attachment factor will be discussed in detail in Section 3.2., where the attachment of vascular endothelium to its underlying substratum will be considered.

2.4. Factor VIII / von Willebrand Factor (F VIII / VWF)

The factor VIII (F VIII) complex may be described as a polydisperse high molecular weight plasma constituent. It is glycoprotein in character and exhibits a range of properties that reflect its essential role in both the coagulation process and the primary haemostatic mechanism (a detailed analysis of this role is given in Chapter 6). More precisely, F VIII isolated as a purified entity from blood, is an association of two different proteins, which can be separated only under certain specific experimental conditions and each of which possesses distinct biological activities which are in accord with the

bifunctional nature of the parent substance (Hoyer, 1981). One of these two proteins can be defined as a high molecular weight species which represents the major protein mass of the whole complex and comprises a series of multimers broadly within the molecular weight range of $1-12 \times 10^6$ (Zimmerman et al., 1975; Counts et al., 1978; Fass et al., 1978; Hoyer and Shainoff, 1980; Ruggeri and Zimmerman, 1980). This protein can be detected by standard immunological techniques using heterologous antisera, as a specific antigenic moiety which has been termed factor VIII-related antigen (F VIIIR : Ag). Structural investigations have indicated that it occurs as a basic assembly of four subunits, each of molecular weight approximately 220 000. These assemblies self-associate to yield a series of polymers, within the molecular weight range described above, reaching an upper limit of approximately 12×10^6. The protein plays an important part in the physiological role of blood platelets and can be identified or measured by its ability to correct certain platelet-functional-defects that occur in its absence or when the protein is defective. Thus, in von Willebrand's disease, in which the protein is either functionally altered or deficient in amount, platelet function is consequently impaired giving rise to the prolongation of bleeding time that characterises this disease (Hoyer, 1976). The disturbance in platelet function can be detected as a defect in platelet adhesiveness as revealed by impaired platelet retention on glass bead columns (Salzman 1963; Bowie et al., 1969) and F VIII can be measured in terms of its ability to correct this defect. FVIII, as detected by demonstration of its ability to restore normal platelet function in von Willebrand's disease, has been referred to as von Willebrand factor (F VIII/VWF). Related to this activity of F VIII, it has been observed that the protein is required as a 'cofactor' in the aggregation of human platelets induced by the antibiotic ristocetin. Plasma from patients with von Willebrand's disease shows a reduced ability to support ristocetin-induced platelet aggregation (Howard and Firkin, 1971). The restocetin co-factor activity of F VIII (F VIII/R : Co) appears to be associated with the largest of the aggregated forms of the protein. It is believed that F VIII/VWF plays an essential role in primary haemostasis in vivo by facilitating the attachment of platelets to the subendothelial surface, following exposure of the latter as a consequence of vascular damage during injury. Defective platelet adhesion is regarded as the basis of the haemostatic defect in von Willebrand's disease. This will be reviewed in further detail later in this chapter when the F VIII-collagen interaction is discussed.

The second protein constituent of the F VIII complex is of relatively low molecular weight, approximately 285 000, as determined by gel filtration of the protein dissociated from the native complex. It exhibits the procoagulant activity of the parent complex, an activity that is absent in classical haemophilia

(haemophilia A) and has been designated therefore as F VIII:C or anti-haemophilic factor (AHF).

2.4.1. Synthesis of Factor VIII/von Willebrand Factor by endothelium

The identification of the endothelium as a possible source of F VIII/VWF was first indicated by immunofluorescence studies with heterologous antisera. These studies demonstrated the presence of F VIIIR:Ag in the intimal region and more specifically the endothelium of the blood vessel wall of both arteries and veins of various sizes and capillaries from a wide variety of organs (Bloom et al., 1973; Hoyer et al., 1973; Holmberg et al., 1974). These immunofluorescence studies served to prove an association of F VIII with the endothelium although they could not in themselves provide unequivocal evidence for its synthesis at this site. More convincing evidence for the actual synthesis of F VIII/VWF by endothelial cells arose from studies which demonstrated the presence of F VIII R:Ag in endothelial cell cultures and its accumulation in the culture medium and especially from the detection of radiolabelled F VIII following incubation of cells with radioactive amino acid precursors (Jaffe et al., 1973; Booyse et al., 1977; Macarak et al., 1977; Shearn et al., 1977; Folkman et al., 1979; Johnson, 1980; Tuddenham et al., 1981) (see also Chapter 6). Synthesis of F VIII/VWF activity in terms of the appearance in culture medium of ristocetin cofactor activity and the ability of culture medium to correct the defective retention of platelets in the blood of von Willebrand patients to glass beads has also been shown (Jaffe et al., 1974; Shearn et al., 1977). Interestingly, the concomitant synthesis of F VIII:C (AHF) by cultured endothelial cells has not been demonstrated. By contrast with these studies, it should be mentioned that Wall et al. (1980) have concluded that the association of F VIII/VWF with the endothelium is attributable to the presence, on the surface of endothelial cells, of high-affinity specific binding sites that permit the adsorption and internalisation of F VIII from blood. These authors consider that the accumulation of F VIII-related activities in culture media arises from the release of F VIII/VWF previously bound to cells prior to their culture. Additionally, Wall and coworkers were unable to measure any synthesis of radiolabelled F VIII following exposure of cultured cells to radioactive precursors. The evidence for specific binding sites has been contradicted by the studies of Jones et al. (1981) who were unable to detect any specific binding of exogenous ^{125}I-labelled F VIII to the surface of cultured endothelial cells. The presence of F VIII on the surface of cells after their isolation and culture, as detected by these latter authors using immunofluorescence, was attributed to non-specific adsorption or alternatively to the presence of F VIII as a membrane constituent.

Thus, several studies have presented evidence for the synthesis of F VIII/VWF by endothelium. Its deposition within the subendothelium where it appears to be closely associated with the basement membrane, has been demonstrated by immunofluorescence following removal of the endothelium (Rand et al., 1980). The role of F VIII/VWF in the interaction of platelets with the subendothelium will be discussed later in this chapter.

2.5. Other glycoproteins derived from the endothelium

2.5.1. Entactin

The isolation, from the matrix deposited in culture by a mouse endodermal cell line, of a basement membrane-associated sulphated glycoprotein of molecular weight 158 000, which is distinguishable from fibronectin and laminin, and which is termed entactin has been described (Bender et al., 1981; Carlin et al., 1981). Immunolocalisation studies have indicated the presence of entactin in close association with a wide variety of basement membranes including that of the vascular subendothelium. The glycoprotein appears to be located in the basement membrane proper, but more especially, in the space between the membrane and its associated cells thus suggesting a possible role for it in cell-matrix interactions. Studies of the distribution of entactin imply its synthesis by both epithelium and endothelium. The synthesis, by cultured endothelial cells, of a sulphated glycoprotein that may conceivably be identical to entactin has recently been reported (Heifetz and Allen, 1982).

2.5.2. Amyloid P-component

Amyloid P-component, a plasma constituent known to occur in amyloid deposits, represents another glycoprotein recently described as of basement membrane origin. Its occurrence in the lamina rara interna of the glomerular basement membrane suggests it may be a product of the endothelium. It also appears to occur in association with the microfibrillar elements of the elastic fibre and it has been shown to form calcium-dependent complexes with fibronectin and C4-binding protein (Dyck et al., 1980; de Beer et al., 1981).

2.5.3. Elastic fibres

Although elastin is not a glycoprotein, it seems pertinent to mention here the presence of elastic fibres as constituents of the vascular subendothelium, that along with collagen fibrils and the basement membrane, are exposed in the event of endothelial denudation and may, therefore, potentially interact with platelets following injury to the vessel wall (Stemerman, 1974).

Production of elastic fibres, including the associated microfibrillar glycopro-

tein component, has been shown morphologically in endothelial cultures (Jaffe et al., 1976) and synthesis of the protein elastin, has been demonstrated by Carnes et al. (1979) and by Cantor et al. (1980). Neither isolated elastin nor its associated microfibrillar protein has been found to possess platelet aggregatory activity. Furthermore, the isolated microfibrillar protein does not appear to be able to bind platelets and binding to elastin does not occur to any great extent (Ordinas et al., 1975; Barnes and MacIntyre, 1979a). It should be noted, however, that in contrast to these findings, others have observed the adhesion of platelets to microfibrillar elements (glycoprotein in character) in the subendothelium, which are exposed upon removal of the endothelium experimentally (Stemerman et al., 1971; Stemerman, 1974; Birembaut et al., 1982). Perhaps pertinent to this is the observation that the cell line BHK-21 and various other cell types can attach, in vitro, to elastic microfibrillar protein via what appears to be a fibronectin-dependent mechanism (Knox et al., 1982).

2.5.4. Thrombospondin

The identification of thrombospondin as a major glycoprotein constituent amongst the products secreted into the medium by cultured endothelial cells has recently been reported (Doyle et al., 1980; McPherson et al., 1981). This glycoprotein also occurs in the α-granules of platelets from which it is released following their stimulation. A role in haemostasis may be anticipated but this has yet to be defined. As a platelet constituent it is believed to play a role in platelet-platelet interactions during platelet aggregation (Jaffe et al., 1982). Its possible presence in the subendothelium has not so far been reported.

2.5.5. Anti-thrombin III

Anti-thrombin III appears to represent another endothelial cell-derived plasma glycoprotein (Chan and Chan, 1981) with an important role related to blood clotting. As an inhibitor of thrombin, it is thought to maintain blood fluidity at the endothelial surface (see also Chapter 6).

3. Interactions at the subendothelium

3.1. The collagen-platelet interaction

The endothelium normally presents a non-thrombogenic surface to the circulating blood. In the event, however, of its damage or some alteration to its integrity, blood platelets can interact with the exposed subendothelial tissue. This may lead to their aggregation and to the formation of mural thrombi. The

aggregation of platelets by elements of vascular (or other) connective tissue following exposure of one to the other as a consequence of injury to the vessel wall, has long been recognised as an important event in permitting the development of the haemostatic plug which leads to the arrest of bleeding (Barnes, 1982). Collagen was identified as the constituent in the extracellular matrix interacting with platelets during early investigations and the demonstration of the ability of collagen fibres to cause platelet aggregation in vitro was first made some twenty years ago (Bounameaux, 1959; Hugues, 1960; Kjaerheim and Hovig, 1962; Zucker and Borelli, 1962; Hovig, 1963). No other isolated matrix constituent of the vessel wall has been found to possess platelet aggregatory activity (Barnes and MacIntyre, 1979a). It is now recognised that through their effect on platelets, collagens in the vessel wall may also promote the sequence of reactions associated with coagulation, the occurrence of which also helps to prevent the escape of blood after injury. The two processes of platelet aggregation and coagulation are intimately related, the one augmenting the other. Thus, they act in concert to affect haemostasis and to localise the events associated with this phenomenon to the site of injury. As a consequence of their ability to interact with platelets collagens can also be regarded as potentially active agents in the induction of thrombosis, especially those collagenous elements that are present in the immediate subendothelial locality and, therefore, most likely to be exposed following a loss or disturbance in endothelial integrity. Furthermore those in the atherosclerotic plaque, exposed when a plaque 'splits' could also be important. A knowledge of the precise platelet reactivity of these particular collagens is a matter of some considerable significance as regards an appreciation of the thrombotic potential of the subendothelium in both 'normal' and diseased (atherosclerotic) blood vessel wall. Activation of platelets by collagens in the vessel wall may also be an important event in the initial stages of atherogenesis. Ross and Glomset (1976) have summarised the evidence for the view that atherosclerosis represents a reponse to injury, involving a sequence of reactions in which following some type of insult to the endothelium, platelets then attach to the wall surface at the site of the endothelial lesion. Damage to the endothelium may conceivably be haemodynamic in nature or may occur as a result of its exposure to toxic elements in cigarette smoke or to high levels of plasma lipoproteins or from some other cause. Platelets adhering to constituents in the subendothelium, presumably collagenous in nature, are stimulated to secrete factor(s) that cause the proliferation of smooth muscle cells that accumulate at the site of injury following their migration from the media. These cells then assume a synthetic activity, and deposit an extracellular matrix which is largely collagenous in nature and which contributes to the substance of the growing atherosclerotic plaque.

3.1.1. The platelet reactivity of individual collagen types

Several laboratories have compared the platelet-aggregating ability of the two interstitial collagen types I and III and there is general agreement that when exposed to platelets in fibrillar form both are potent aggregatory agents. When presented in solution (i.e. as the monomeric species tropocollagen) it has been commonly observed that collagen type III is very much more potent than type I. However, preincubation of the solution (in platelet-poor plasma, for example) at 37°C or prior dialysis against a low-ionic strength phosphate buffer (procedures both known to promote the formation of native-type collagen fibrils of 67 nm periodicity) leads to an increase in the aggregatory activity of each collagen and the large difference in activity previously noted is no longer apparent. These observations are entirely in accord with the concept that collagen(s) can only induce platelet aggregation if they are in fibrillar form. The enhanced activity of type III, as compared to type I, when presented to platelets in solution must presumably reflect a more ready ability of collagen type III to form fibrils in platelet-rich plasma (Balleisen et al., 1975; Barnes et al., 1976a; Hugues et al., 1976; Santoro and Cunningham, 1977). Since both collagens possess a comparable aggregatory activity as fibres both are likely to play a role in haemostasis. Furthermore because both are located in the subendothelium (at least of the ageing vessel wall, where some degree of diffuse intimal thickening can be expected) and in the intimal plaque they must each be regarded as being of importance in the context of thrombosis.

Initial studies with collagens types IV and V suggested that these collagens were unable to induce platelet aggregation (Barnes and MacIntyre, 1979a, 1979b; Trelstad and Carvalho, 1979; Barnes et al., 1980). Thus, solutions of these collagens, even following pretreatment under conditions known in other cases to induce the formation of highly active fibrils of 67 nm periodicity, failed to exhibit any platelet aggregatory activity. However, it seemed very likely that this inactivity was due to the absence of a suitable fibrillar structure rather than any inability of collagen types IV and V to aggregate platelets per se. These collagens are not thought to occur as fibrils of 67 nm periodicity in vivo and difficulty in generating this type of fibril from their solutions in vitro has been a common experience. In order to assess the influence of quaternary structure on the platelet reactivity of collagens type IV or V, Barnes et al. (1980) investigated the activity of other possible polymeric forms, namely segment-long-spacing (SLS) aggregates in which the molecules are aligned laterally in a head-to-head, tail-to-tail arrangement. Collagen types IV and V were found to form this type of structure under the same conditions used to generate SLS aggregates of the interestitial collagens. Such structures were found, like those of type I collagen (Muggli, 1978; Wang et al., 1978), to

induce the aggregation of platelets (see Fig. 5.2), thus further emphasising the importance of the quaternary structure in relation to the ability of any given collagen to cause platelet aggregation. Prior to testing, SLS aggregates were stabilized by formaldehyde treatment and this produced end-to-end polymerisation of aggregates to yield forms which the authors in question consider may represent the true aggregating species (see Wang et al., 1978). Subsequently, other polymeric forms of these two collagens, including type V-derived fibrils of 67 nm periodicity, produced by prolonged dialysis of a type V solution against physiological saline at 25°C, and non-striated fibrils of type IV collagen (from human placenta) were found to possess platelet aggregatory activity (Barnes et al., 1980; see Fig. 5.3). Others have also independently reported the ability of collagen types IV and V in a fibrillar form to aggregate

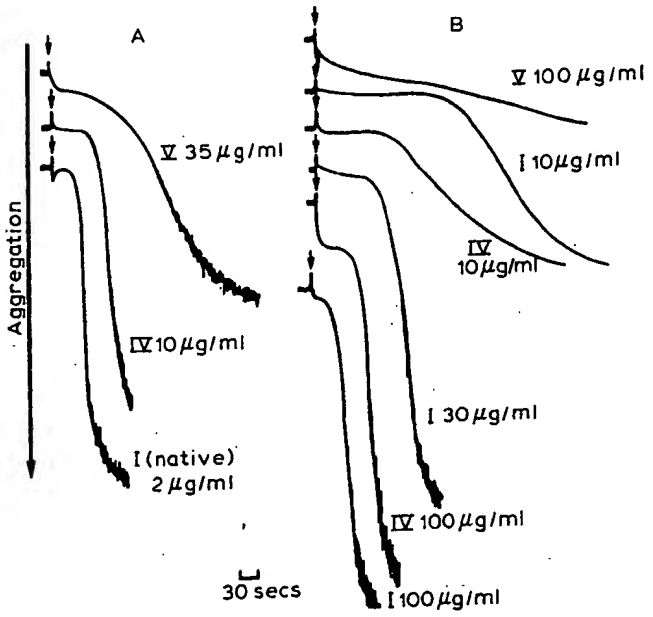

Figure 5.2. Platelet aggregation by SLS polymers of collagens types I, IV and V.

The results of two separate experiments are shown. Activity was measured at the concentrations specified. (A) Aggregation by SLS forms of Types IV and V collagen (from human placenta) and by native-type fibrils of Type I (from bovine tendon); (B) Aggregation by SLS forms of Types IV and V collagens from human placenta and Type I from rat tail tendon (SLS aggregates of Type I collagen from human placenta behaved similarly to those of Type I from rat tendon). Reproduced with permission from Barnes et al., 1980 (*Thromb. Res.* 18, 375–388).

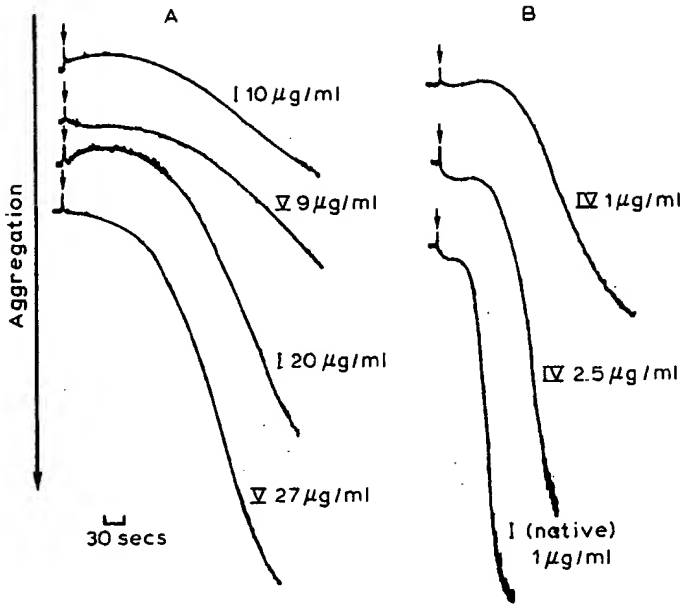

Figure 5.3. Platelet aggregation by collagens types IV and V.

(A) Type I and Type V collagen fibrils of 67 nm periodicity formed by dialysis of solutions of these collagens from human placenta against physiological saline at 25°C; (B) non-striated fibrils of Type IV collagen (from human placenta) formed by dialysis of a solution against 0 02 M Na_2HPO_4 compared to native-type fibrils of Type I (from bovine tendon). Activity was measured at the concentrations specified. Reproduced with permission from Barnes et al., 1980 (*Thromb. Res.* 18, 375–388).

platelets (Balleisen and Rauterberg, 1980; Chiang et al., 1980; Tryggvason et al., 1981).

Clearly then, collagen types IV and V, like the interstitial collagens, have the ability, when they assume an appropriate quaternary structure, to interact with platelets to cause their aggregation. The question remains, what is the platelet reactivity of these two collagens in the subendothelium? Their existence as fibrils of 67 nm pediodicity in vivo has not been proven (fibrils of this nature are not observed in basement membranes) and their precise quaternary structure remains unknown. The possibility exists that these collagens may occur in the subendothelium in a form that is unable to induce the aggregation of platelets. They may, rather, permit platelet adhesion to the subendothelium in the absence of aggregation. Baumgartner and his co-workers (Baumgartner,

1977; Baumgartner and Muggli, 1976) have described the interaction of platelets with the subendothelium of rabbit aorta exposed experimentally by use of a balloon catheter. Following attachment to the subendothelium platelets underwent aggregation but the aggregates then rapidly dispersed leaving, essentially, a monolayer of cells covering the surface. The ability of platelets to adhere in this manner to the subendothelial surface, following its exposure by alteration in the integrity of the overlying endothelium, has been noted in a number of other instances (Majno and Palade, 1961; Movat and Fernando, 1963; Tranzer et al., 1968; Ts'ao and Glagov, 1970; Schwartz and Benditt, 1973). For example, platelets are seen to fill the gaps between endothelial cells induced by the use of reserpine (Tranzer and Baumgartner, 1967). It is believed that this phenomenon may be of possible importance, in maintaining endothelial integrity in vivo. Baumgarter (1977) considered that the basement membrane is responsible for the adhesion of platelets in these situations. Others have advanced a possible role for elastic fibres and their associated microfibrillar elements, both of which have been identified as components of the subendothelial surface (Stemerman, 1974; Birembaut et al., 1982). However, neither of these structural elements reveal much platelet binding activity when in an isolated form (Ordinas et al., 1975; Barnes and MacIntyre, 1979a). Baumgartner has observed that following treatment of the subendothelium with α-chymotrypsin, which removes basement membrane (and the microfibrillar component of the elastic fibre), the total surface coverage with platelets is reduced, although at sites where adhesion does occur aggregation proceeds more readily. This has been attributed to the greater accessibility of interstitial collagen fibrils in the subendothelium to platelets following chymotrypsin treatment. The precise platelet reactivity of basement membranes, e.g. anterior lens capsule or glomerular basement membrane, has been the subject of some uncertainty, but it would seem that these structures, when examined as intact entities, may permit adhesion in the absence of aggregation (Huang et al., 1974; Freytag et al., 1978; Huang and Benditt, 1978; Barnes et al., 1980). This adhesion has been attributed to a noncollagenous glycoprotein (Huang and Benditt, 1978). Nevertheless, the present authors consider that the possibility still remains that collagen types IV or V may play a role in the adherence of platelets to the subendothelium. Of particular interest in this context is the observation of Balleisen and Rauterberg (1980) that collagen type IV, 'short-chain' collagen and the type V-derived A-chain, although in a form unable to induce platelet aggregation could, however, promote the spreading of platelets on plastic (Zaponlack).

The precise platelet reactivity of the subendothelium and the identification of the components involved in the interaction remain important issues, particu-

larly with regard to thrombosis. How far this disease may involve the straightforward aggregation of platelets by interstitial collagens exposed through injury, or, alternatively, the exposure of a surface that normally permits only adhesion but that in the disease has become altered so that aggregation can occur (reflecting either a change in collagen composition or in platelet reactivity of collagen(s) not normally able to induce platelet aggregation), has still to be resolved.

3.1.2. The role of intermediary proteins in the collagen-platelet interaction

3.1.2.1. Fibronectin. As discussed elsewhere in this chapter, fibronectin is a product of the endothelium which has been shown by immunofluorescence procedures to occur in the subendothelial matrix. At this site it may play an important role in cell-cell and cell-matrix interactions, including those involved in the attachment of endothelial cells to their underlying substratum. This glycoprotein, which is known to form complexes with collagens and other types of molecule, occurs in the blood as a soluble component and has also been shown to occur in platelets where, following their stimulation, it is in part released (Zucker et al., 1979a) and in part accumulates on the platelet surface (Ginsberg et al., 1980). Platelets stimulated by thrombin have also been shown to bind plasma fibronectin (Plow and Ginsberg, 1981). Such observations have led to the consideration that fibronectin may be involved in the process of platelet adhesion, either to non-platelet surfaces or in platelet-platelet interactions (platelet aggregation). Bensusan and his colleagues (Bensusan et al., 1978) have proposed that this protein may mediate in the interaction between collagens and platelets. They found, for example, that following sonication of a mixture of platelets and collagen, fibronectin could be detected bound to the collagen moiety. Furthermore, preincubation of collagen with fibronectin inhibited the collagen-platelet interaction. In support of the possible involvement of fibronectin in platelet adhesion, a number of authors has described the ability of this protein to enhance the attachment and spreading of platelets on collagen and gelatin substrata (Hynes et al., 1978; Grinnell et al., 1979; Koteliansky et al., 1981). Others however, have questioned any role for fibronectin in the collagen-platelet interaction. Sochynsky et al. (1980) concluded that since inhibition of platelet adhesion to collagen by preincubation of the latter with fibronectin was achieved only after a preincubation period of some minutes, this was too slow for it to be considered likely that fibronectin participated in the adhesion process, in vivo, which by comparison was a very much faster event. In accord with this conclusion, Cohen et al. (1981) have demonstrated that the presence of exogenous fibronectin has no effect on the rate or extent of platelet aggregation induced by a variety of agents, including

collagen. Exposure of platelets to anti-fibronectin antibodies has been shown to have little effect on the adhesion of platelets to collagen (Santoro and Cunningham, 1979; Sochynsky et al., 1980). Furthermore, preincubation of platelets with gelatin, which is believed to interact with fibronectin much more effectively than native collagen (see Section 2.3.) did not effect adhesion to any great extent and did not prevent aggregation (Santoro and Cunningham, 1979). This argues against the participation of fibronectin on the platelet surface in the collagen-platelet interaction. It should be noted, however, that Bensusan et al. (1978) found that platelet fibronectin interacted more readily with native collagen than gelatin. The precise role of fibronectin in the collagen-platelet interaction thus remains obscure and it has been argued (Sochynsky et al., 1980) that in view firstly of the inhibition of platelet adhesion to collagen following preincubation of the latter with fibronectin and secondly because it is possible that collagens in the subendothelium are saturated with fibronectin, the latter may even serve to discourage, rather than promote, interaction of platelets with the subendothelium.

3.1.2.2. Laminin. In view of the identification of the glycoprotein laminin as a constituent of basement membranes, it is not unreasonable to consider the possible involvement of this protein in the adherence of platelets to the subendothelial basement membrane. Laminin has been reported to mediate the preferential attachment of isolated epithelial cells to a collageneous substratum composed of the basement membrane collagen, type IV (see Section 3.2). Although laminin possesses no platelet aggregatory activity, nor is it able to inhibit or prevent the aggregation of platelets by interstitial collagens (Tryggvason et al., 1981), its ability to actively promote the collagen-platelet interaction or to facilitate the adhesion of platelets to basement membrane has not, so far, been specifically investigated.

3.1.2.3. Factor VIII/von Willebrand factor. The role of plasma F VIII/VWF in primary haemostasis is believed to be one of facilitating or promoting the interaction of platelets with the subendothelial surface. Thus Baumgartner and his colleagues (Baumgartner et al., 1977, 1980) have observed that platelets in blood from patients with von Willebrand's disease show defective binding to the exposed subendothelium of rabbit aorta. Platelets from normal individuals, after treatment of the blood with anti-F VIII/VWF antibodies, show an even more greatly impaired adhesion. The defect is only obvious at high shear rates, such as those appertaining to capillary flow. However, if the subendothelial surface is treated with chymotrypsin, which removes the basement membrane and the microfibrils associated with elastic fibres thereby accentuating the exposure of collagen fibrils, then the attachment of platelets to the latter is defective even at the relatively low shear rates,

normally occurring in large blood vessels. Furthermore, aggregation of platelets (once attachment has occurred) also appears to be inhibited. The studies of Sixma and his associates (Sakariassen et al., 1979; Bolhuis et al., 1981) have also illustrated a defective binding of platelets to subendothelium when F VIII/VWF is deficient. These authors, by using ^{125}I-labelled F VIII/VWF, have been able to demonstrate that the attachment of platelets is preceded by an attachment of F VIII/VWF and have also observed, like Baumgartner and colleagues, that F VIII/VWF promotes the spreading of attached platelets. These observations may also be consistent with those of Booyse et al. (1981) who have described a decreased interaction between isolated platelets and the matrix produced in culture by endothelial cells derived from the aorta of pigs with von Willebrand's disease.

This evidence for the mediation of F VIII/VWF in the reaction of platelets with a subendothelial surface, including their interaction with collagen fibres at this site, suggests that F VIII/VWF and collagen may directly bind or adhere one to the other, serving thereby to promote the interaction between platelets and collagen. Indeed, evidence to this effect has been presented by investigators from a number of different laboratories. Nyman (1977, 1980) has studied the interaction between F VIII/VWF and a number of different commercial collagen preparations largely, if not wholly, type I in composition. Fibres reconstituted in vitro from solutions of commercial preparations of water- or acid-soluble calf-skin collagen were found to adsorb F VIIIR : Ag from plasma with the apparent concomitant dissociation of F VIII : C activity from the F VIII complex. By contrast, fibre suspensions from commercial preparations of 'insoluble' collagen (comprising native collagen fibres originally formed in vivo) failed to interact with F VIIIR : Ag. Legrand et al. (1978) reported that fibres reconstituted from a solution of highly purified pepsin-solubilised type III collagen from calf skin were very much more active than similar fibres from type I collagen, which exhibited relatively poor adsorption of F VIIIR : Ag. Type III fibres did not cause dissociation of the F VIII : C activity. This might imply that the activity observed by Nyman (1977, 1980) was due to the presence of some type III collagen in the commercial preparations used. However, Santoro (1981) has reported that reconstituted native-type fibres of highly purified human collagens, types I, II, and III obtained by pepsin digestion, all adsorbed F VIIIR : Ag and FVIII R : Co from plasma very effectively, with some concomitant release of the F VIII : C activity. In all of these studies the interaction between collagen and F VIII has been demonstrated by incubation of a suspension of collagen fibres in plasma and measurement of FVIII-related activities following the subsequent removal of collagen fibres by centrifugation. In studies in the authors' laboratory, the

binding of F VIII/VWF to a variety of collagens has been examined by measuring the adsorption of purified ^{125}I-labelled F VIII to air-dried collagen films prepared by allowing collagen solutions in dilute acetic acid to evaporate to dryness. Using this technique we and our colleagues (Scott et al., 1981) have found comparable binding of F VIII by the interstitial collagens, types I and III and by basement membrane collagen type IV and the basement membrane-associated collagen, type V (see Fig. 5.4). Binding was found to be

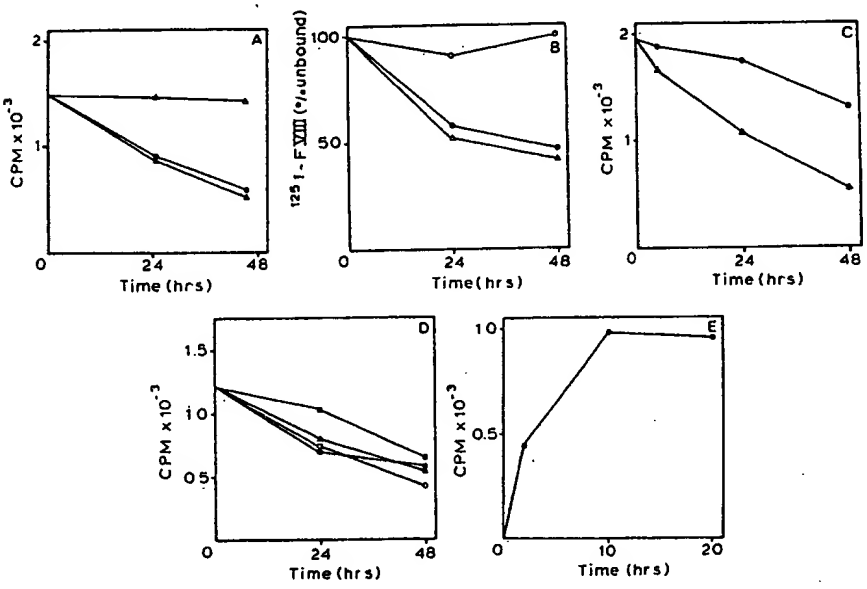

Figure 5.4. Interaction between F VIII / VWF and collagens.

(A–D) Binding of ^{125}I-F VIII/VWF to air dried collagen films. Binding is indicated by a decrease in radioactivity in the F VIII/VWF solution. (A) △———△, untreated wells; ●———●, wells treated with 6 μg collagen (bovine tendon type I fibres); ▲———▲, wells treated with 60 μg collagen. (B) ●———●, wells treated with 2 μg collagen (human placenta type I); ○———○, wells treated with 2 μg collagen and assayed in the presence of a 25 fold excess concentration of unlabelled F VIII/VWF; △———△, wells treated with 2 μg collagen and assayed in the presence of a 10 fold molar concentration of fibronectin relative to that of F VIII/VWF. Results in this case are expressed as the percent of ^{125}I-F VIII/VWF unbound to the collagen films. (C) ▲———▲, 6 μg of collagen (bovine tendon type I fibres); ●———●, 6 μg of denatured collagen (10 min at 100°C). (D) ●———●, type I collagen; ▲———▲, type III; ■———■, type IV; ○———○, type V. All collagens were from human placenta. Wells were treated with a solution containing 2 μg of the appropriate collagen. (E) Binding of ^{125}I-F VIII/VWF to bovine tendon collagen fibres in suspension. ^{125}I-F VIII/VWF binding is indicated by the increase of ^{125}I associated with collagen fibres. Reproduced with permission from Scott et al., 1981 (Thromb. Res. 24, 467–472).

unaffected by the presence of fibronectin suggesting separate binding sites on the collagen molecule for fibronectin and F VIII/VWF. Binding to native collagen was found to be more effective than to the denatured form, gelatin. We have also examined the binding of ^{125}I-labelled purified F VIII/VWF to a suspension of type I collagen fibres using a very finely dispersed preparation of native bovine tendon fibres. In contrast to the data of Nyman (1977, 1980) we found these native fibres, formed in vivo, absorbed F VIII/VWF rapidly, saturation being achieved in ten minutes or less (Fig. 5.4).

Consistent with these investigations demonstrating that an interaction between collagen and F VIII may be important in promoting platelet activation by collagen, Baugh et al. (1979) have reported that preincubation of a solution of collagen (from calf or guinea pig skin) with bovine F VIII/VWF, prior to its addition to a platelet suspension, causes collagen-induced aggregation at concentrations of collagen below those required for aggregation when collagen alone is added. An enhanced platelet-aggregatory activity of insoluble collagen fibres (equine) was also noted and in this case preincubation of collagen and F VIII was not necessary. In addition, Morin et al. (1980) have noted a decreased adhesion of platelets, in vitro, to collagen fibrils suspended in platelet-rich plasma prepared from the blood of patients with von Willebrand's disease.

The role of the F VIII/VWF actually contained within the platelet (as opposed to that occurring in the F VIII complex in plasma) in the collagen-platelet interaction is unclear but there is some evidence that it may be important in the platelet-platelet interactions that occur following the adhesion of platelets to collagen (Baumgartner et al., 1980). It has also been proposed however that it may be important in the binding of F VIII : C (Zucker, 1979b).

3.2. The roles of fibronectin and laminin in cell-matrix interactions

Fibronectin has been demonstrated to promote the attachment and subsequent spreading of a variety of cell types on artificial substrata such as plastic or glass (Pearlstein, 1976; Juliano and Gagalang, 1977; Pena and Hughes, 1978; Grinnell and Minter, 1978; Grinnell, 1976; Grinnell and Hays, 1978; Benedetto et al., 1981; Harper and Juliano, 1981), or on an extracellular matrix component such as collagen (Klebe, 1974; Klebe et al., 1977; Pena and Hughes, 1978; Kleinman et al., 1978; Linsenmayer et al., 1978; Murray et al., 1979; 1980; Chicquet et al., 1979; Gold and Pearlstein, 1980; Berman et al., 1980; Rich et al., 1981; Schor et al., 1981). Fibronectin has also been implicated, at least in part, in the attachment of metastatic melanoma cells to the basal lamina of cultured endothelial cells (Nicholson et al., 1981) and of transformed fibroblasts (PyBHK cells) to blood vessel subendothelium (Pearlstein and Hoffstein, 1981).

The actual mechanism by which fibronectin acts as an attachment intermediary is thought to involve the initial binding of fibronectin to collagen (Klebe, 1974; Kleinman et al., 1978; Pearlstein, 1977). This, it is suggested, may result in a conformational change in the fibronectin molecule (Pearlstein, 1977), which is followed by the binding of the matrix-fibronectin complex to the cell surface. This latter process requires the presence of calcium and magnesium ions (Klebe et al., 1977) and is an energy dependent step (Juliano and Gagalang, 1977; Shiba and Kanno, 1977). Other components of the extracellular matrix have been suggested to participate in this fibronectin mediated cell adhesion. Hyaluronate and heparan sulphate (Jilek and Horman, 1978; Stathakis and Mossesson, 1977; Yamada et al., 1980), for example, have been shown to stabilise fibronectin-collagen binding and it is possible that the relatively low level of fibronectin binding to native collagen may be due to the absence of additional participatory macromolecules such as proteoglycans which may promote the interaction (Jilek and Horman 1978; Johansson and Hook 1980).

There have, however, been a number of reports in which certain cell strains such as WI-38, MRC-5 and human conjunctiva cells (Taylor, 1961; Witkowski and Brighton, 1971; Rajaraman et al., 1974), have been shown to attach and spread in the absence of exogenous fibronectin. The present authors have found this to be the case also with endothelial cells. We have been studying the attachment of porcine aortic and bovine corneal endothelial cells with the purpose of gaining some insight into the factors controlling the binding of the endothelium to its underlying substratum. We have found that these cells attached to and spread on a variety of collagen types with the vascular cells binding very much more effectively than those from the corneal endothelium (Fig. 5.5). Cells from the latter source consistently revealed slower binding to type V collagen. The binding of endothelial cells from both sources to collagen type I was consistently more rapid than to other collagen types but by contrast with epithelial cells they exhibited no preference for basement membrane (type IV) collagen. The attachment or spreading of both endothelial cell types to these collagens was generally independent of the addition of exogenous fibronectin. This finding is in contrast to the studies of Gold and Pearlstein (1980), who reported that human umbilical cord endothelial cells were completely dependent on the presence of exogenous fibronectin for their binding to collagen (type I). It has been shown that normal diploid cells generally secrete higher levels of fibronectin than established or transformed cell lines (Vaheri and Ruoslahti; 1975; Mosher, 1977; Yamada et al., 1976; Olden and Yamada, 1977). Furthermore, cells maintained in serum-free medium (and therefore in the absence of exogenous binding factors such as fibronectin) are known to

deposit a substantial sub-cellular matrix (Yaoi and Kanaseki, 1972; Pegrum and Maroudas, 1975) that may conceivably be involved in their attachment. Consequently, it has been suggested by Grinnell (1978) and subsequently shown by Grinnell and Feld (1979) that certain cell types such as fibroblasts are capable of binding to, and spreading upon, fibronectin (or a fibronectin-like protein) secreted by the cells themselves and deposited on to the underlying substratum. In accord with this suggestion we have also found in our studies, using antiserum specific for bovine fibronectin, that the attachment of bovine corneal endothelial cells (and possibly by implication other endothelial cells) to collagen (type I) appears largely to involve endogeneous fibronectin (Fig. 5.6.). This observation is consistent with the synthesis of high levels of fibronectin by endothelial cells (see Section 2.2.4.).

It has also been suggested that some cell types may only recognise collagen in certain physical conformations, although the interpretation of some of these results is somewhat controversial. Studies using three dimensional native collagen gels have indicated that cell adhesion to these gels is independent of fibronectin, even in the case of cell lines that have otherwise been shown to exhibit either a partial (Linsenmayer et al., 1978; Kleinman et al., 1979; Harper and Juliano, 1981) or a complete (Grinnell and Minter, 1978; Rubin et al., 1978; 1981a; 1981b; Schor and Court, 1979; Grinnell and Bennett, 1981; Schor et al., 1981) dependence on fibronectin for their binding to either air-dried native collagen films, or to gelatin-coated surfaces. Thus, it may be that cells may interact with native collagen gels without fibronectin involvement and bind directly to collagen via a membrane bound receptor, as has been described for fibroblasts with type I collagen (Goldberg, 1979) and for smooth muscle cells with type V collagen (Grotendorst et al., 1981). Consequently, the view has been expressed that cell adhesion to collagen may not require fibronectin in vivo, but that under certain circumstances, the presence of fibronectin in specific regions of the connective tissue matrix may modify adhesive interactions.

The second major glycoprotein which has been implicated in cell-collagen attachment is laminin, a large glycoprotein which has been shown to occur in association with various membranes, including those of the blood vessel endothelium (see Section 2.2.1.) where it may play an important role in the adhesion of endothelial and possibly other cell types. Epithelial cells from various sources (Murray et al., 1979; Hughes et al., 1979; Wicha et al., 1979; Terranova et al., 1980) have been shown to bind poorly to the interstitial collagens but show a preference for type IV collagen by a mechanism not stimulated by serum or fibronectin. Terranova et al. (1980) have shown that at least for the PAM 212 epithelial cell line, and possibly other epithelial cells,

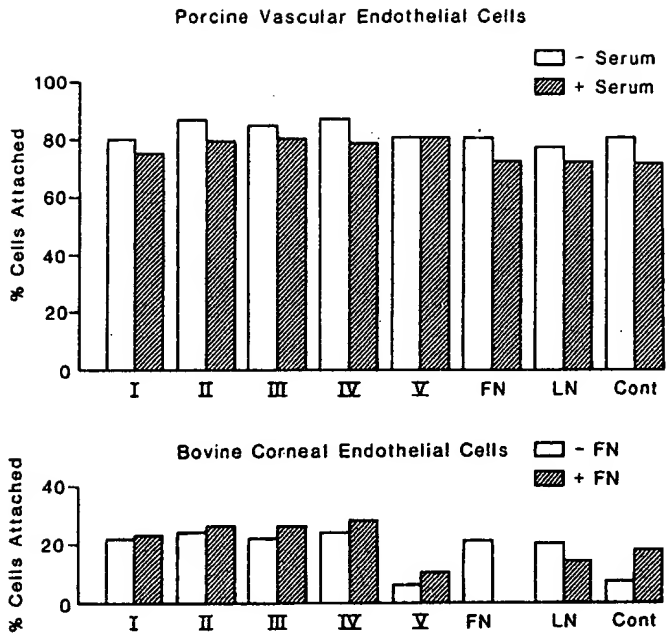

Figure 5.5. Attachment of vascular and corneal endothelial cells to collagen and other connective tissue proteins.

Air-dried films of native collagen (types I to V), fibronectin (FN) or laminin (2.9 μg of protein cm^{-2}) were prepared as described by Kleinman et al. (1979) and preincubated for 90 min at 37°C with either: (a) Dulbecco's Minimum Essential Medium (DMEM) alone □, or (b) DMEM containing 10% (v/v) foetal calf serum or FN (25 μg ml^{-1}), ▨. Confluent monolayer cultures of porcine aortic or bovine corneal endothelial cells were trypsinised to form monodisperse cell suspensions. The cells were then allowed to attach to the protein-coated surfaces for 60 min in the presence of BSA (25 μg ml^{-1}). Unattached cells were then washed off and counted electronically, and from this value the percent of attached cells determined (unpublished data; Scott, D.M., Murray, J.C. and Barnes, M.J.).

this binding to type IV collagen is mediated by laminin. The mechanism whereby laminin binds cells to type IV collagen is thought to be similar to that described for fibronectin, in that the molecule ininitially binds to type IV collagen and the cell then binds to the collagen-laminin complex. Johansson et al. (1981) have shown that rat hepatocytes are capable of binding equally well to either laminin or fibronectin. We have also shown that porcine vascular and bovine corneal endothelial cells (Fig. 5.5) and smooth muscle cells (data not

shown) are also capable of attaching effectively to either laminin or fibronectin. Thus it would appear likely that cells, such as endothelial cells, which are capable of synthesising both laminin and fibronectin (Gospodarowicz et al., 1981; see section 2.3.3. and 2.2.4), may attach to a variety of collagen types utilising mechanisms involving fibronectin and/or laminin. Such characteristics may be important in the repair of damaged endothelium when it is possible that any of the collagens of the subendothelium may be exposed.

Recently, the ability of tumour and other cell types to bind to endothelial cells and their basal lamina has been studied (Vladovsky and Gospodarowicz, 1981; Nicholson et al., 1981; Pearlstein and Hoffstein, 1981). Metastatic melanoma cells were shown to bind (although poorly) to cultured endothelial cell monolayers (which do not show apical surface fibronectin) via a non-fibronectin binding mechanism, although they bound to the basal lamina synthesised by endothelial cells by a process at least partially mediated by fibronectin (Nicholson et al., 1981). Pearlstein and Hoffstein (1981) have also shown that the binding of transformed fibroblast cells to the subendothelium (authentic blood vessel subendothelium) was not exclusively via fibronectin. These studies thus indicate that metastatic cells probably bind to the subendothelial basal lamina via a variety of adhesive mechanisms and that each class of adhesive interaction may, individually, play only a partial role, but collectively they play a major role in the successful blood-borne arrest and extravasation of these cells, or the enhancement of their implantation and subsequent establishment as secondary tumours.

Other protein molecules have also recently been suggested to play a role in the binding of certain cell types to collagens. For example a large serum borne glycoprotein (which has also been shown to be secreted by chondrycytes) termed chondronectin has been implicated in the binding of chondrocytes to type II collagen (Hewitt et al., 1980). Fibronectin however, appears to be involved in the spreading of these cells once bound. Recently two other collagen binding proteins of lower molecular weights(70000 and 58000) have been shown to be synthesised by a number of normal and malignant adherent cells (Vartio and Vaheri, 1981), and rat hepatoma cells (Dickey and Seals, 1981) respectively. Whether such proteins play a role in the attachment of cells to the subendothelium is not known.

Considering that the vascular endothelium is subjected to the severe hydrodynamic forces of the blood it is important that the vascular endothelial cells are anchored securely to the underlying substratum in order to maintain, amongst other properties, the non-thrombogenic nature of the blood vessel wall. In view of the evidence for the involvement of fibronectin and laminin (both of which are found in detectable amounts in, or in association with, the

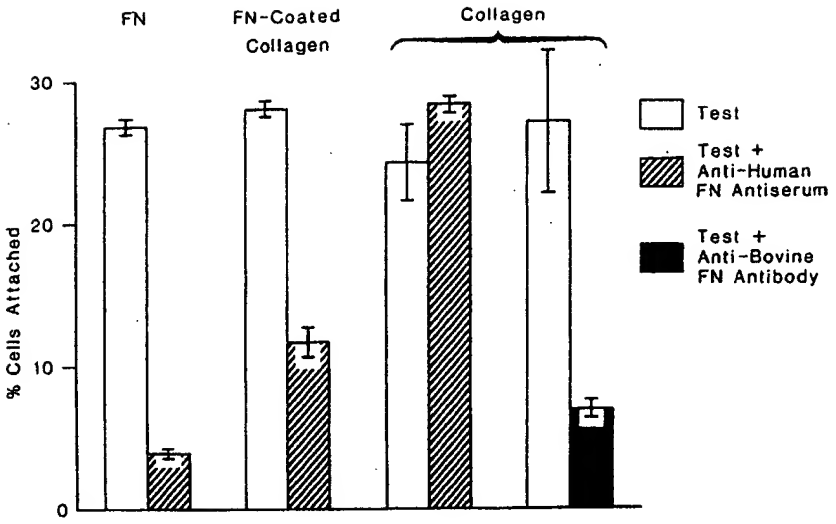

Figure 5.6. Effect of anti-fibronectin antiserum and antibody on endothelial cell attachment to collagen and fibronectin.

Air-dried collagen films (collagen type I) were prepared as described in Fig. 5.5. Appropriate surfaces were coated with human FN by incubation (90 min, 37°C) with a solution (25 $\mu g\ ml^{-1}$) of the protein. Confluent monolayer cultures of bovine corneal endothelial cells were trypsinised to form monodisperse cell suspensions. The cells were then allowed to attach (in the presence of BSA, 25 $\mu g\ ml^{-1}$) for 60 min to either human FN, air-dried collagen or air-dried collagen coated with human FN, in the absence (indicated as test in the figure) or presence of rabbit anti-human FN antiserum (10%, v/v) or rabbit anti-bovine FN antibody (54 $\mu g\ ml^{-1}$). Cells prior to attachment were incubated for 45 min at 37°C in either DMEM (test sample) or DMEM containing anti-FN antiserum or antibody as indicated in the figure. Anti-human FN antiserum although effectively inhibiting cellular attachment to exogenous human FN did not prevent binding to untreated (FN-free) collagen surfaces. However binding to the latter appeared to be largely mediated by endogenous FN since it was markedly inhibited by anti-bovine-FN antibody (Unpublished data; Scott, D M., Murray, J.C. and Barnes, M.J).

subendothelial basement membrane) in the cell-attachment process, it is likely that one of the major functions of these molecules is to provide strong anchorage of endothelial cells to the subendothelium. Following damage and loss of endothelial integrity it is possible that these molecules may have an important part to play in endothelial repair (see also Chapter 13), although it should also be borne in mind that their possible interactions with other cell

types such as macrophages, platelets or tumour cells may perhaps play a role in the pathogenesis of vascular disease.

4. Summary

The arterial subendothelium is generally recognised as containing a number of morphologically distinct elements including collagen fibres, elastic fibres with their associated microfibrillar structures and amorphous material constituting the basement membrane. Amongst the variety of products that have been identified in a subendothelial matrix as constituents of, or as associated with, these different entities can be listed a number of glycoproteins including (1) collagens types I and III (the interstitial collagens) which occur in fibrous form, collagen type IV which is located in the basement membrane and collagen type V which is also closely associated with this structure; (2) fibronectin, laminin and other non-collagenous glycoproteins such as entactin and amyloid P-component, which are either constituents of the basement membrane or appear to be closely associated with it; and (3) glycoproteins associated with the elastic fibre, including the microfibrillar element and amyloid P-component, already referred to as a constituent of the basement membrane.

Most, if not all, of these proteins can be synthesised by cells derived from the endothelium in vitro and the origin of at least some of these proteins in the subendothelium can reasonably be regarded as being primarily from this source in vivo. Other components of the subendothelium, such as type I collagen, may perhaps be regarded as being derived from smooth muscle cells that have migrated into the intimal space during the course of intimal thickening, than from endothelial cells. The relative importance of the one cell type as compared to the other in the synthesis of any particular individual constituent of the subendothelium is in many cases as yet unclear, since both cell types can synthesise many of the proteins described above in vitro.

As products of the endothelium, some of these proteins also occur in the circulation, such as fibronectin (which exists in plasma as a soluble product commonly designated cold-insoluble globulin or plasma fibronectin), and amyloid P-component. Factor VIII/von Willebrand factor represents a further example of a glycoprotein produced by the endothelium that occurs in the plasma, as part of the factor VIII complex, and has also been identified in the subendothelium.

The precise structural and functional inter-relationships between these proteins as regards their possible role in the correct functioning of the

circulatory system or in such processes as repair of the damaged vessel wall, or those involved in the prevention of loss of blood at sites of injury, have yet to be fully elucidated. This chapter has considered two aspects of such inter-relationships, firstly in the response of platelets to the subendothelial surface following its exposure as a consequence of injury and secondly in the attachment of the endothelium to its underlying substratum.

The aggregation of platelets by fibrillar collagens is regarded as an important event in primary haemostasis and the interaction of platelets with collagenous elements in the subendothelium following exposure of the latter as a result of damage to the endothelium may be an important aspect of thrombosis and atherosclerosis. As summarised in this chapter, there is considerable evidence to support the proposal that factor VIII/ von Willebrand factor plays an important intermediary role in the interaction of platelets with collagen in the blood vessel wall and the ability of this protein to bind to a variety of collagen types has been demonstrated. The role of other glycoproteins, such as fibronectin, in the collagen-platelet interaction is, however, less certain. The possibility is discussed that collagens types IV and V may interact with platelets differently to the fibrillar interstitial collagens (types I and III) allowing the adhesion of platelets to the subendothelial surface without the formation of aggregates.

The integrity of the endothelium is essential to maintain the non-thrombogenic nature of the luminal surface of the vessel wall. There are good reasons for supposing that both fibronectin and laminin may be involved in the attachment of the endothelium to an underlying collagenous matrix. In accord with this supposition, isolated endothelial cells have been found to attach to a variety of collagens in vitro, in a process largely mediated by endogenous fibronectin, and the ability of these cells to bind to laminin has also been noted.

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